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Regulation of Cellular Proliferation Effects on Alteration of Normal Signaling Pathways

David Malkin, MD

INTRODUCTION

The transformation of a normal cell into a malignant one requires the stochastic acquisition of multiple genetic alterations. These involve the inactivation, or loss of function, of negative growth regulatory genes known as "tumor suppressor genes," in addition to the activation of growth promoting genes known as "oncogenes." The functional interaction of these altered genes and their respective protein products under the influence of other regulatory proteins leads to changes in the cell's ability to respond appropriately to external signals in the form of growth inhibitory peptides. Ultimately, unchecked cell division and proliferation lead to neoplastic transformation.

The characteristic pattern of regulation of cell proliferation has been defined as the cell cycle. Malignant cells have lost the ability to regulate the cell cycle negatively and therefore continue to divide in an uncontrolled fashion. An increase of positive growth signals derived from mutated proto-oncogenes can override this negative growth control; on the other hand, inactivation of negative growth signals can drive through the checkpoint controls at different points in the cell cycle. The best understood of these checkpoints are found at the initiation and completion of DNA synthesis (S phase) and of cell division (mitosis [M]). The cyclic regulation of the cell cycle is determined by the periodic accumulation and degradation of proteins known as cyclins during the distinct phases of the cycle. The expression of cyclins during these specific times in the cell cycle leads to the activation of cyclin dependent kinases that complex with the cyclins to regulate their expression.

In the presence of damage to DNA, the importance of cell cycle regulation becomes particularly relevant, for the replication of a cell with damaged genetic material would presumably lead to the proliferation of cells destined to malignant transformation and the persistence of malignant clones. In fact, checkpoints are deregulated during oncogenesis, often as the result of changes in cyclin-cyclin dependent kinase complex formation.

This article summarizes our current understanding of the role of the distinct families of genes and proteins involved in cell cycle regulation, and discusses the interaction of these factors in the setting of mutagenic events that arrest cell division and permit DNA to repair itself. The effects of alterations of these normal regulatory signaling pathways will be addressed. The association of these various cell cycle regulatory elements is shown in Figure 1.

THE CELL CYCLE

Tumor Suppressor Genes and the Cell Cycle

p53. Alterations of the p53 tumor suppressor gene are the most frequently encountered genetic events in human malignancies [1–3]. The gene, located on the short arm of human chromosome 17 band 13 encodes a 53 kDa nuclear phosphoprotein that appears to function as a negative regulator of cell growth and proliferation [4–5]. The gene contains 11 exons. Analysis of the nucleotide and amino acid sequences demonstrates five phylogenetically conserved domains among vertebrates. These regions are regarded as being essential for the functional activity of p53. Several properties of the p53 protein are indicated by the presence of two simian virus (SV40) large tumor-antigen (T-Ag) binding sites, a nuclear localization signal, an oligomerization domain, and several phosphorylation sites [4,5].

The p53 protein was initially identified in SV40-transformed mouse fibroblasts where it was thought to be a transformation-specific protein due to its apparent interaction with the T-Ag of SV40 virus [6,7]. This virus, found in monkeys, is a member of the polyomavirus family. These viruses encode viral T-Ag, which are synthesized immediately after infection. The proteins are responsible for the loss of control of cell growth that is induced by the virus both in vitro and in vivo. Transforma-

From the Divisions of Oncology and Immunology & Cancer Research, Department of Pediatrics, The Hospital for Sick Children, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada.

Address reprint requests to Dr. David Malkin, Division of Oncology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

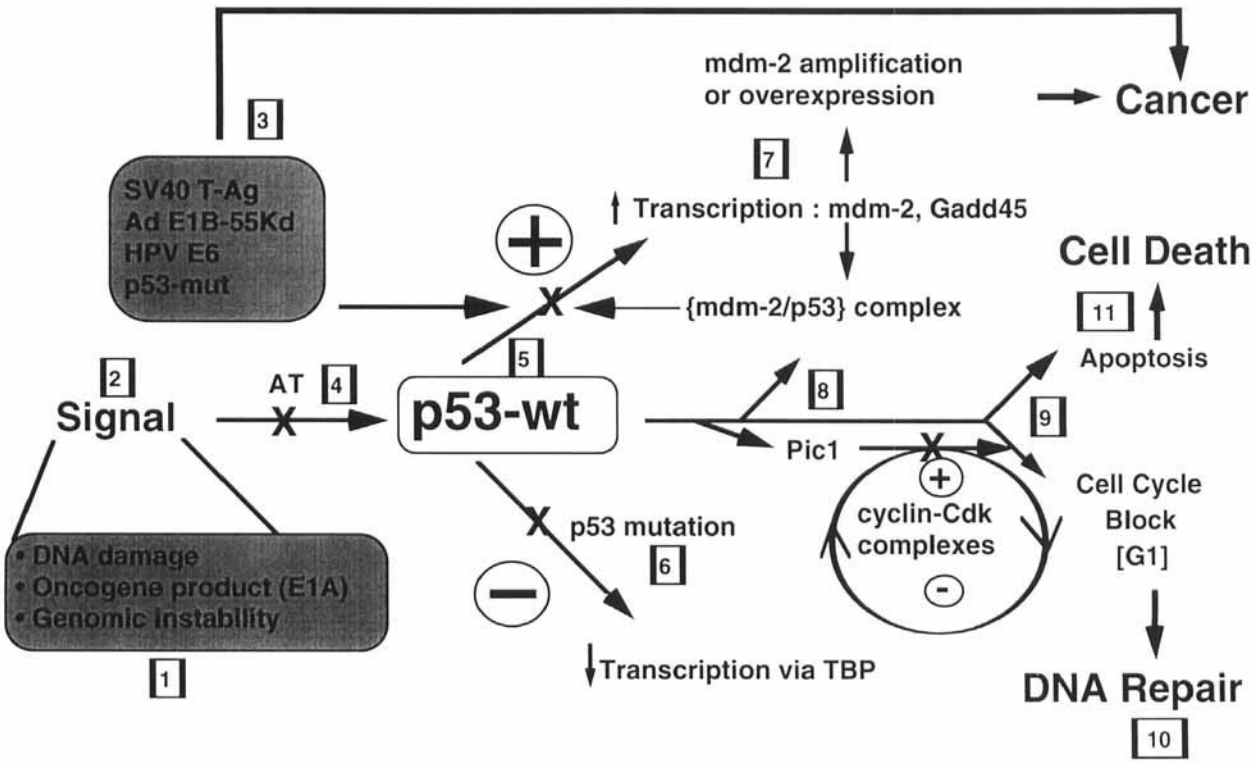

Fig. 1. Regulation of cellular proliferation. Various events (1) that induce genomic alterations lead to transduction of a signal (2) that ultimately guides the cell through the cell cycle. Although the presence of wild-type p53 (5) is critical to the integrity of cell cycle regulation, the presence of normal ataxia-telangectasia gene(s) (4) are also believed to be important in stabilization of the genome, and probably play an important role in controlling p53 function. The occurrence of p53 alterations disrupts its function and can lead to decreased transcription of other growth regulatory elements (6) via transcription binding proteins. p53 Mutations themselves or infection with oncogenic viruses including SV40, adenovirus, or human papillomarvirus (3) could directly lead to cellular transformation and malignancy. Indirectly, these alterations might induce expression of positive growth regulatory elements such as mdm-2 or Gadd45, leading to malignant transformation (7). Normal p53 induces Pic1 which is involved in cell cycle arrest at G1/S phase. The complex interaction of the cyclins, cyclin dependent kinases and Cdk inhibitors ultimately drives the cell to cell cycle arrest allowing DNA repair to occur (10), or to apoptotic programmed cell death (11).

tion assays in rat fibroblast cells have demonstrated that only mutant forms of p53 could transform and immortalize, while the wild-type protein actually suppressed transformation [8]. This observation, coupled with the fact that p53 is frequently mutated or lost in many human tumors suggests that the normal function of p53 is that of a "growth suppressor."

The p53 protein binds specific DNA sequences and appears to be a transcription factor that may regulate the expression of other genes. The introduction of wild-type p53 protein blocks the growth of many transformed cells [9,10], most likely by blocking progression of cells through the cell cycle late in the G1 phase of cell replication. In addition, wild-type p53 could be involved in restricting precursor cell populations by mediating apoptosis, or programmed cell death, in the absence of appropriate differentiation or proliferation signals. Cells that either lack p53 gene expression or overexpress mutant p53 do not exhibit G1 arrest. The fidelity of DNA repair during cell cycle arrest may play a role in the capacity of cells to tolerate radiation injury and therefore have an impact on radiation sensitivity. It has been reported in this context that p53 may play a role in the cellular response to gamma-irradiation damage through DNA synthesis inhibition, following DNA damage, and thereby provides a cell cycle "checkpoint" [11–14].

Inactivation of p53 function occurs by several mechanisms. The occurrence of missense mutations, deletions, or nonsense mutations of the gene prevents the protein from forming tetrameric complexes that bind specific DNA sequences. All mutant forms of p53 lose the ability to bind p53-binding sites and cannot therefore activate expression of downstream growth-regulatory (reporter) genes [15]. Conformational changes of a mutant p53 molecule can affect wild-type molecules complexed with the mutant form within the tetramers preventing the complex from binding to DNA and transcriptionally activating reporter genes. Inactivation of the p53 protein through binding of other cellular proteins may also prevent normal binding and thus prevent transcriptional activation. Some

of the DNA tumor virus genes, including SV40 T-Ag, the E1B gene of adenovirus, and the E6 gene of human papilloma virus, encode proteins that bind to p53. In cells that co-express one of these proteins along with p53, expression of p53-inducible reporter genes cannot be activated. This inhibition of expression may be critical for viral replication and/or cell transformation. Disruption of normal p53 function may also be altered by alteration of MDM2, a cellular gene that was originally identified by virtue of its amplication in a spontaneously transformed mouse cell line. The MDM2 gene product has been shown to bind to p53, resulting in inhibition of the ability of p53 to transactivate genes adjacent to p53-binding sites. This gene appears to be amplified in a significant fraction of human sarcomas; the consequent overexpression of MDM2 probably interferes with p53 activity [16].

The ability of p53 to arrest cell growth either through overexpression or through a G1 DNA damage pathway may in part be explained by its ability to induce other growth regulatory genes downstream of it. In particular, it is now understood that progression of cells through the cell cycle is governed by the sequential formation, activation, and subsequent inactivation of a series of cyclin-dependent kinase (Cdk) complexes. Positive regulation of the cell cycle by cyclin-Cdk complexes, and negative regulation at certain checkpoints monitor the initiation and completion of DNA synthesis and formation of a functional mitotic spindle. One recently isolated protein variously known as Cip1, Sdi1, and Waf1 (Pic1), has been shown to be a potent inhibitor of cyclin-Cdk complex kinase activity, with an ability to associate with several different cyclins and cyclin complexes. Upon stimulation of Waf1 expression by wild-type p53, Waf1 could contribute to growth arrest through late G_1 into S phase. The loss of normal p53 activity in tumors might lead to decreased expression of Pic1 and subsequent failure to arrest in G1 in response to appropriate DNA-damaging stimuli [for review see 17,18].

Although the precise function(s) of p53 are not clear, the following model may explain many of the observations described above. For normal cell division, p53 is not required. However, in the response of a normal cell to DNA damage, p53 levels rise and the cell is blocked in G_1, thus allowing it to undergo genomic repair or apoptosis. In those cells in which the p53 pathway is inactivated by gene mutations, or by host or viral oncoprotein interactions, damaged DNA is replicated. This results in mutation, aneuploidy, mitotic failure, and cell death. Malignant clones may arise from the survivors of the "genetic scrambling" and lead to cancer formation.

pRB. The retinoblastoma susceptibility gene maps to human chromosome 13q14. The gene, RB1, which was initially isolated as a 4.7 kilobase cDNA fragment from retinal cells consists of 27 exons and encodes a 105 kDa nuclear phosphoprotein. As well as being altered in retinoblastoma, this gene and its protein product have also been found to be altered in osteosarcomas, small cell lung, bladder, breast, and prostate carcinomas. It is thought that reduction to homozygosity of the mutant allele (or loss of heterozygosity [LOH] of the wild-type allele) leads to loss of functional RB1 and accounts for tumor development.

Although it is clear that RB1 and its protein product play some role in growth regulation, the precise nature of this role remains obscure. In the developing retina, inactivation of the RB1 gene is both necessary and sufficient for tumor formation. Although the RB1 gene is expressed in virtually all mammalian tissues, only in the retina is its inactivation sufficient for tumor initiation. Outside the retina, RB1 inactivation is often a rate-limiting step in tumorigenesis generated by multiple genetic events.

Structural characteristics of the RB1 protein suggest that it is an ubiquitous nuclear phosphoprotein involved in transcriptional control. It appears that this tumor suppressor protein has binding domains that indicate it interacts with a variety of substrates including viral transforming oncoproteins such as the E1A protein of adenovirus 12, as well as the Myc family of oncogenes. Recent experiments have also led to the identification of a family of at least seven cellular proteins that are able to interact with the SV40 large T/E1A-binding domain of pRB with the same specificity as T and E1A. These observations suggest that cells produce proteins that have the potential of interacting in a physiologically relevant manner with pRB. It is possible that the RB1 gene product is inactivated in retinal cells by the complex that forms between it and these other compounds [19].

The growth suppressing function of RB1 may also be related to its cyclical changes in phosphorylation during the cell cycle. Although the relative amounts of p105Rb remain essentially constant during the cell cycle, the protein is in its unphosphorylated form in the G0 and G1 phases, and becomes phosphorylated by late S phase and throughout mitosis. In rapidly proliferating cells, the RB1 protein is highly phosphorylated. It is evident that p105RB in some way regulates cell cycle progression and cell growth and differentiation. When altered or absent, controlled cell growth would be ablated [20]. A complete outline of the role and functions of RB1 are discussed by Yandell and Poremba, elsewhere in this supplement.

CYCLINS AND CYCLIN DEPENDENT KINASE COMPLEXES

Checkpoints exist at the G_1/S transition point of the cell cycle for three particular purposes. These include: 1) the prevention of replication of DNA damaged by external factors including gamma-irradiation or certain chemotherapeutic agents; 2) the provision of time for

DNA repair to occur and enhance the likelihood of cell survival; and 3) the prevention or limitation of propagation of heritable genetic errors. As indicated previously, p53 and pRB are critical to G_1/S checkpoint arrest.

The cyclins and cyclin-dependent kinaes (Cdk) are early regulators of cell cycle progression [21,22]. The control of Cdk function enables the control of multiple downstream mediators of gene expression in the growth regulatory pathway. The kinase activity is regulated at four distinct levels either through the expression of a specific regulatory subunit, preferential cdk/cyclin complex formation, phosphorylation, or active inhibition through binding of other inhibitory proteins.

The association of Cdks with their respective regulatory subunits retains some specificity. For example, cyclins A and B associate with cdc2 (cdk1), cyclin D associates with cdk4, and cyclins A and E form complexes with cdk2. This differential subunit association is, in part, responsible for the regulation of kinase activity. Cyclins D and E are activated in G_1 and prior to the G_1/S transition, cyclin A is unregulated at the exit of S phase, and cyclin B at the G_2/M transition phase [23].

Cyclin dependent kinases appear to function primarily by exerting a positive growth control during the cell cycle. This commonly occurs by inactivation of negative growth regulators best exemplified by its effect on pRB. As previously indicated, the association of pRB with the transcription factor E2F maintains pRB in its dephosphorylated state and induces cell cycle arrest both at the G_1/S transition and the G_2/M transition phases. On the other hand, in the presence of an external growth signal that stimulates cyclin dependent kinase activity, pRB is hyperphosphorylated leading to deregulation of E2F and inactivation of the growth inhibitory state. Thus, the cell is permitted to progress through these checkpoints [24].

It has become evident most recently that cdk activity is significantly regulated by the interaction of the cdk-cyclin complex with cyclin-dependent kinase inhibitors, and that multiple cdk inhibitors control the cell cycle. For example, the complex of cdk4 with cyclin D is active in regulating the G1 phase of the cell cycle. Stimulation of the cdk inhibitor p16 induces binding of this protein with the cdk4-cyclin D complex, yielding an inactive state and cell cycle arrest at the G_1/S transition. Presumably, alterations of the p16 protein perturb this inactivation. The association of cdk2 with either cyclin A or cyclin E may be inhibited by the expression of several cdk inhibitors including p21, p27, and p28. Many of these cyclin-cdk-cdk inhibitor associations are still poorly understood, although at least one is more clearly established as outlined below.

It appears that DNA damage by gamma-irradiation or certain chemotherapeutic agents including the epipodophyllotoxins induce G_1/S arrest by induction of p21. This cdk inhibitor blocks cyclin-mediated activation of transcription factors, most notably by preventing hyperphosphorylation of RB and the subsequent lack of induction of E2F. Thus, S-phase genes fail to be transcribed. In normal G_1/S arrested cells, p21 exists as a component of a quaternary structure with its complementary cyclin dependent kinase-cyclin complex and the DNA replication and repair factor proliferating cell nuclear antigen (PCNA). It is likely the disruption of p21 from this complex that yields the dysregulation of the cell cycle and permits the cell to enter S phase [25].

CONCLUSIONS

All cells undergo a regulated wave of activity of many proteins during cell cycle progression. The cyclins and their catalytic partner proteins, the cyclin dependent kinases, are particularly suited to their significant role in regulation of progression through the cell cycle. The cyclins are thought to determine the subcellular localization, the substrate specificity, the interaction with upstream regulatory enzymes, and the timing of activation of the cdks. Several pieces of evidence implicate cyclins in the deregulation of cell division observed in malignant cells. In addition to the increasing attention given to the cyclins and cdks in the normal and deregulated control of the cell cycle, one must maintain close attention to the pivotal role played by the tumor suppression genes, particularly p53 and pRB. Their direct involvement in cell cycle regulation indicate that proteins that negatively regulate the cell cycle must be inactivated as well. Whether the cell cycle inhibitors ultimately are proven themselves to be tumor suppressors is still a matter of speculation.

Wild-type p53 functions as a negative regulator of cell growth and tumor suppression and may play a significant role in genomic instability and DNA repair. Mutant p53, in its various forms, can abolish many of these activities. Abnormalities of p53 appear virtually ubiquitously in human cancer. Despite this, the multiple inactivation mechanisms of upstream and downstream regulators of p53 function; and, perhaps even the potential of certain cell subtypes to bypass the p53-regulatory pathway, will make it difficult or even preclude the development of universal chemotherapeutic or gene-based therapeutic approaches to the treatment of human cancer related to p53 dysfunction. Perhaps, however, induction of mutant p53 to its wild-type counterpart, or selective elimination or inactivation of mutant p53 in affected tumor cells may lead to viable therapeutic options.

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